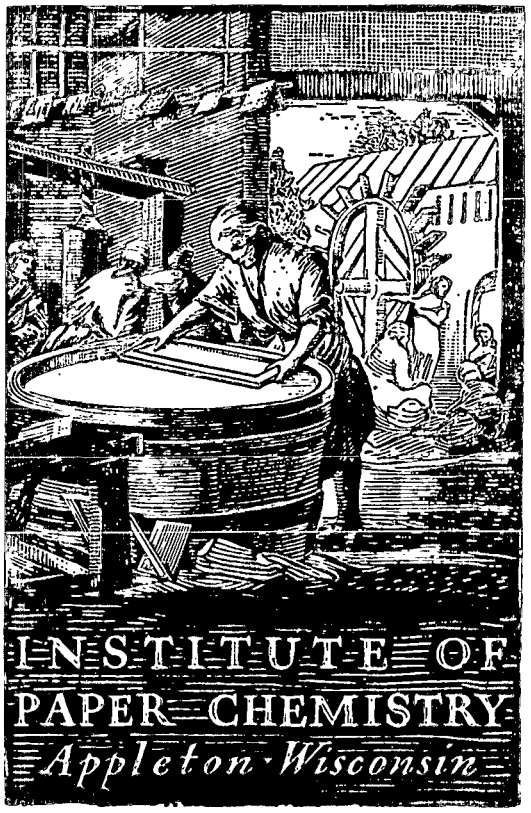

AN EVALUATION OF THE FATHEAD MINNOW RESIDUAL OXYGEN ASSAY (ROA) AS AN ACUTE TOXICITY INDICATOR FOR PULP AND PAPER MILL EFFLUENTS AND EFFLUENT COMPOUNDS

Project 3355

Report One
A Progress Report
to
MEMBERS OF THE INSTITUTE OF PAPER CHEMISTRY

March 19, 1982

THE INSTITUTE OF PAPER CHEMISTRY

Appleton, Wisconsin

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THE INSTITUTE OF PAPER CHEMISTRY

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SUMMARY

The residual oxygen assay is an alternative acute toxicity bioassay which measures the amount of oxygen remaining in a sealed jar, containing a fish, following the death of the fish. Aqueous materials such as mill effluents and leachates can be tested over a range of concentrations. Acutely toxic concentrations will result in the death of the fish before all of the dissolved oxygen in the medium is used up. Thus, the amount of residual oxygen is indicative of the relative toxicity of the test stream.

This assay has an elapsed time of 4-8 hours compared with the 96-hour conventional bioassay and could provide a short-response, simple, and sensitive toxicity test for mill or laboratory application. This test can be done easily on site by mill personnel at very low cost.

The objective of this study was to evaluate the application of this test to a warmwater test fish, the fathead minnow. All previous work has been done with salmonid fish, which are cold-water species. Also evaluated were operating parameters which affected results, reproducibility, relationship to the LC50, and the availability of sublethal information from the procedure.

Separate and identifiable responses occurred for toxic and nontoxic materials. Interference problems occurred for effluents with a great deal of BOD and not enough toxicity to inhibit bacterial activity. This caused declines in oxygen not caused by the fish. An experiment was conducted to test UV light as a pretreatment to reduce microbial activity. The UV treatment was effective, but

only where light penetration was adequate or when effluents were diluted to reduce turbidity and color.

The elapsed time to the death of the test fish was explored as a possible sublethal response parameter for effluents giving a nontoxic response. Significant elapsed times between controls and high concentrations always occurred for toxic effluents and occasionally for nontoxic effluents. Various differences in initial oxygen levels or test temperatures also occurred, and no conclusive relationship between elapsed time and sublethal toxicity was established. Elapsed time for the entire experiment could be reduced by testing under warm temperatures and at high fish loading rates without affecting the sensitivity of the test.

Oxygen uptake rates were also investigated as a possible sublethal response parameter; they were not significantly different even for acutely toxic materials if temperature and fish weights were held constant.

Types of effluents tested did not appear to affect results though most (but not all) treated effluents were nontoxic, and no untreated mill effluent produced a nontoxic response.

The ROA showed an overall correlation with LC50 data generated by 96-hour exposure static assays with $r^2 = 0.848$. For a subsample made up of the effluents from mills ($n = 4$), an r^2 of 0.99 was obtained. A larger sample of toxic mill effluent comparisons would be desirable, but the indications are that good correlation exists between the ROA and LC50.

The residual oxygen assay shows great promise as a supplement to conventional bioassays. It has research and mill applications and could be especially useful as a monitoring tool or to quickly and inexpensively generate large data bases for practical applications.

INTRODUCTION

Conventional laboratory fish toxicity bioassays require an exposure period of 96 hours or more according to currently acceptable practices (1-2). If acute bioassays are being conducted as a management tool to evaluate effluents, leachates, spills, process changes, or experimental formulations, this elapsed time may be inconvenient or impractical. The long duration increases the likelihood of accidents and complications. In research it is desirable to generate numerous replications to ensure statistical confidence in the conclusions obtained. Often experimental conditions may change before adequate data can be collected from long-exposure assays. In addition, the 96-hour exposure test is complicated, requiring skilled personnel and elaborate facilities.

For these and other reasons, a bioassay that can produce results in less than 8 hours is desirable and useful. In 1969 Ballard and Oliff (3) proposed a bioassay method for marine fish which required exposing fish to varying concentrations of a material in sealed containers. Following the death of all the exposed fish the residual oxygen was measured and concentrations with more oxygen than the control were identified as toxic response levels.

In 1976 McLeay applied this test to measuring pulp mill effluent with salmonid fish and found it to be a sensitive and useful tool (4). Vigers and Maynard (5) refined the test somewhat in 1976 by using standard BOD bottles and expressing the results as a threshold limit (TLM) extrapolated from a log-log plot of the residual oxygens.

To date this test has been utilized only with salmonid and marine fish species. Since there is evidence that differences in fish species affect the results

(6), an evaluation of a warmwater species was felt to be desirable for possible use in U.S. mills located in warmwater areas. The residual oxygen assay was evaluated as part of the work of Project 3355, which was initiated in the Environmental Sciences Division and funded by the Institute's membership. The objective of this work was to determine whether the ROA could be used with a warmwater species, the fathead minnow, for U.S. pulp mill effluent. At the same time the limitations and applications of the test were to be explored as well as the applicability of this test to measure sublethal stress effects.

METHODS AND MATERIALS

RESIDUAL OXYGEN ASSAY

The sealed jars used for this assay were conventional 320-mL BOD bottles with tapered necks to accommodate a dissolved oxygen meter probe. The meter used was a Yellow Springs Instrument Company unit and probe with stirrer.

Fish were native adult fathead minnows (*Pimephales promelas*) for the most part although smaller juvenile fish were occasionally used. Fish were obtained locally from minnow dealers and were nearly all live-trapped wild fish from unknown strains. Fish were held at ambient water temperatures and fed a mixture of trout fingerling pellets and HiProMin flake food. If bioassays were run at temperatures different from holding temperatures, the fish were acclimated for at least an hour and occasionally overnight. Fish were not fed on the day of the experiment.

The water source was Appleton City water dechlorinated by activated carbon. Appendix I summarizes pertinent descriptive parameters for the dilution water. Temperature was controlled by a water bath unless the experiments were conducted at room temperature, in which case a water bath was not used.

The procedure followed was to make up a volume of 6-10 different concentrations by serial dilution of the material to be tested. This volume was aerated by vigorous stirring and transferred to 11 numbered BOD bottles. Initial dissolved oxygen, pH, temperature, and time of day were recorded. A data form developed to facilitate this task is included in Appendix II. Fish were added singly to 10 of the 11 bottles, all of which were capped. The fish were left undisturbed until no gill movements could be observed, then the time was noted, and final dissolved oxygen, temperature, and pH were recorded. Dead fish were

weighed singly prior to disposal. Controls consisted of one set of 10 bottles which contained only plain dilution water and fish. In addition each effluent concentration included one blank bottle which contained effluent and no fish. The blank bottle was measured at the time of death of the last fish.

The residual oxygen values were averaged for each group of ten fish and plotted on log-log paper against concentration to obtain a curve for the determination of the threshold level value (TLV). This value is the concentration at which toxicity begins to exert an effect on the residual oxygen by killing the fish before oxygen is used up to the level of oxygen remaining in plain water. At several places in this report statistical evaluations were conducted by linear regression and Student's t test according to Snedecor (7).

In Appendix III a detailed step-by-step procedure is outlined which can be used by someone with basic laboratory skills to conduct a residual oxygen assay (ROA).

ACUTE FISH BIOASSAYS

For comparative purposes some LC50 values obtained from conventional acute toxicity assays are included with this report. Most of these values were obtained by a 96-hour static bioassay using fish and water as previously described. Aeration was supplied by compressed air through inverted funnels only during bioassays where effluent oxygen consumption indicated a need for aeration. Ten fish were exposed to five serial dilutions and a control in 20-liter containers. Temperature, dissolved oxygen, pH, and conductivity were monitored daily. Fish were not fed during the experiment, and weights were obtained for fish that died during the 96-hour exposure. LC50 values were calculated either by graphical methods or by using the Litchfield Wilcoxin probit method. Normally two replications per assay were done.

Not all effluents tested by ROA were tested by the conventional static bioassay. For this reason comparisons for all data cannot be made.

EFFLUENTS

Effluents were collected from a variety of sources and locations largely in conjunction with other projects or investigations. By combining activities a wider range of materials could be subjected to the ROA for a more diversified test of its application. Most effluents were tested fresh or stored in the dark at 4°C. Most mill effluents were transported to the laboratory by air or truck within 24 hours of collection and tested fresh.

Laboratory effluents were generated by pulping, bleaching, and biologically treating (where treated) under laboratory conditions using established practices. Normally those effluents were also produced for purposes other than ROA testing and include some nonconventional processes.

RESULTS AND DISCUSSION

During the evaluation of the residual oxygen assay, sixty-two experiments were conducted to produce information about the toxicity of an effluent or to produce information for evaluating the test itself. A wide variety of biologically treated and untreated mill and laboratory generated effluents was used, as well as pure compounds known to be toxic. For some of the effluents and compounds conventional fish bioassays were also conducted.

A summary of the effluents used during this project is included as Table I. A detailed summary, which includes mean data for each assay, is attached as Appendix IV.

The specific sets of data used to illustrate the response characteristics of this test are taken from the collection of assays.

I. RESPONSE CHARACTERISTICS

Threshold Limit Value (TLV)

The ROA is a dose response test whereby an end point or response level can be determined for a specific point in a range of concentrations. This response level is the concentration at which toxicity begins to influence fish respiration and oxygen consumption. It is expressed by an elevation in the dissolved oxygen which remains in solution following the death of the test animal. The concentration at which toxicity begins to influence residual oxygen levels is the Threshold Level Value (TLV or TL). This point is determined graphically by plotting residual oxygen against effluent concentration on log-log paper and determining the break point in the slope of the two lines formed (see Fig. 1).

TABLE I

SUMMARY OF EFFLUENT TYPES ASSAYED BY RESIDUAL OXYGEN METHOD

	Biologically Treated	Untreated	Total
Mill effluents			
Kraft unbleached	6	3	9
Kraft bleached	5	2	7
Sulfite bleached	2	4	6
Kraft unbleached lab treated	2		2
Lab effluents			
Soda bleached	4	2	6
Soda borate bleached	2	1	3
Borax and additive bleached		1	1
Sulfite bleached		1	1
Kraft bleached	4	4	8
Pure compounds			
Dehydroabietic acid		9	9
Pentachlorophenol		6	6
Alum and water		2	2
Polymer and water		2	2
Total			62

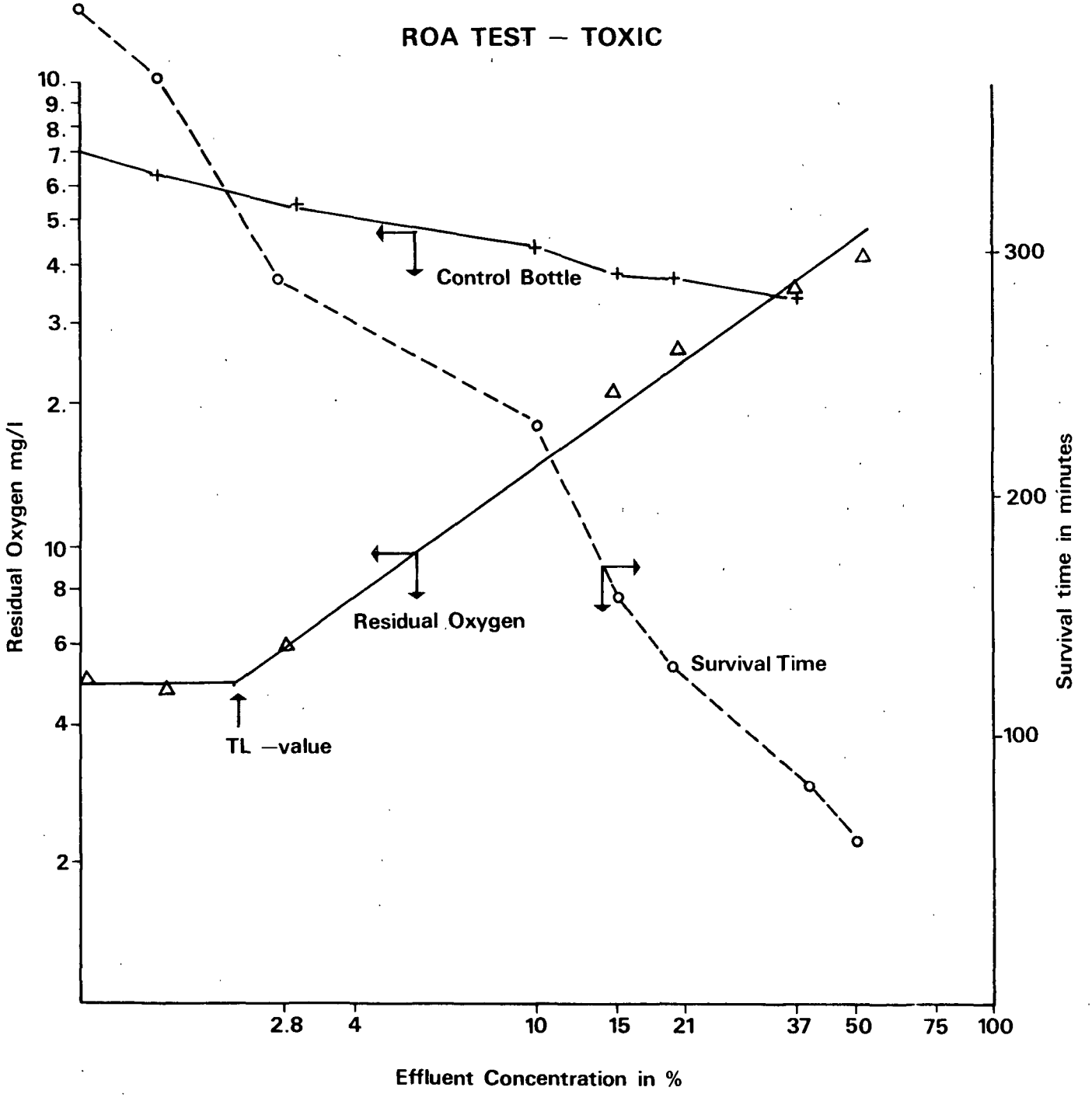

Figure 1. ROA curves for a representative toxic effluent.

Each residual oxygen assay generates 11 data points (10 fish and a blank) per concentration for 8-10 concentrations for each of the variables which include oxygen consumed or remaining, elapsed time, and oxygen uptake rates. A set of data generated for a typical toxic mill effluent is presented in Table II.

TABLE II

DATA GENERATED FOR A TOXIC MILL EFFLUENT
BY THE RESIDUAL OXYGEN ASSAY

Effluent Conc., % vol.	$\bar{x}$ Residual Oxygen, mg/L	Start Temp., °C	$\bar{x}$ Elapsed Time, min	$\bar{x}$ Fish Wt./Bottle, g	$\bar{x}$ Fish Loading, g/L	$\bar{x}$ O_2 Uptake Rate, mg O_2/g fish/min
0 - Control	0.54	20.1	445	1.28	4.14	0.013
Blank[a]	7.1	20.1	485	0	0	--
1%	0.48	20.1	366	1.39	4.50	0.039
1 Blank	6.1	20.1	470	0	0	--
2.8%	0.6	20.1	289	1.47	4.76	0.014
Blank	5.4	20.1	354	0	0	--
4%	0.49	20.1	353	1.18	3.83	0.15
Blank	--	20.1	--	0	0	--
10%	1.54	20.5	227	1.60	5.18	0.133
Blank	4.50	20.5	258	0	0	--
15%	2.32	21.0	163	1.14	3.9	0.022
Blank	3.78	21.0	180	0	0	--
21%	2.62	21.2	139	1.02	3.29	0.026
Blank	3.7	21.2	148	0	0	--
37%	3.5	21.9	82	1.14	3.69	0.030
Blank	3.4	21.9	119	0	0	--
50%	4.1	22.0	65	1.03	3.34	0.049
Blank	4.4	22.0	73	0	0	--

[a]Blank - test solution without fish.

The mean residual oxygen values from column 1 in Table I are plotted on logarithmic paper to form the curves which produce the threshold value. Figure 1 shows the plotted values. The point of deflection of the residual oxygen curve is the TLV, which is determined to be 2.2% for the data in Table I. This is a typical response for a toxic compound or effluent.

A nonacutely toxic effluent will produce a data set similar to that included in Table III. The curves for these data are illustrated in Fig. 2. For this effluent all fish died of asphyxiation when the dissolved oxygen level reached 0.32 to 0.51 ppm. The response of fish in 100% effluent was not different from that of fish in the control water, and no acute toxicity effects were observed for these data.

For both of the data sets presented, a control bottle or blank was used to determine the oxygen consumption of the effluent without a fish being present. The oxygen in the blank is measured at the time of death of the last fish. If necessary, a correction can be made to compensate for oxygen consumed by the effluent; however, this is not necessary for calculating the TL value since the oxygen remaining, not the oxygen consumed by the fish, is used to calculate the TL value.

In both examples previously used, effluent BOD had no influence on the TL value. However, some of the untreated effluents tested had a significant BOD and a source of microbes which combined to rapidly deplete oxygen levels in the jars during the test. Table IV is a data set for an effluent in which oxygen consumption by the effluent resulted in no residuals following the death of the fish. This may indicate a nontoxic response or a false reading due to the BOD. Because these responses cannot be separated, high oxygen consumption in the blank invalidates the

TABLE III

DATA GENERATED FOR A NONTOXIC MILL EFFLUENT BY THE RESIDUAL OXYGEN ASSAY

Effluent Concentration	$\bar{x}$ Residual Oxygen, ppm	Start Temp., °C	$\bar{x}$ Elapsed Time, min	$\bar{x}$ Fish Wt./Bottle, g	$\bar{x}$ Fish Loading, g/L	$\bar{x}$ O_2 Uptake Rate, mg O_2/g fish/min
0	0.45	21	493	1.82	5.88	0.0108
Blank	11.2	21	606	--	--	--
4%	0.51	21.5	511	1.80	5.84	0.0110
Blank	10.2	21.5	606	--	--	--
10%	0.49	21	484	1.72	5.56	0.0118
Blank	10.3	21	580	--	--	--
15%	0.32	21.1	431	1.73	5.62	0.0118
Blank	9.8	21.5	555	--		
21%	0.32	21.2	445	1.58	5.14	0.0125
Blank	9.3	21.0	585	--	--	--
37%	0.40	21.4	383	1.54	5.05	0.0155
Blank	9.3	21.5	465	--	--	--
50%	0.37	21.1	343	1.61	5.20	0.0160
Blank	8.75	21.2	385	--	--	--
75%	0.32	21.2	287	1.68	5.47	0.0166
Blank	8.0	21.2	360	--	--	--
100%	0.42	21	239	1.76	5.71	0.0177
Blank	7.4	21	360	--	--	--

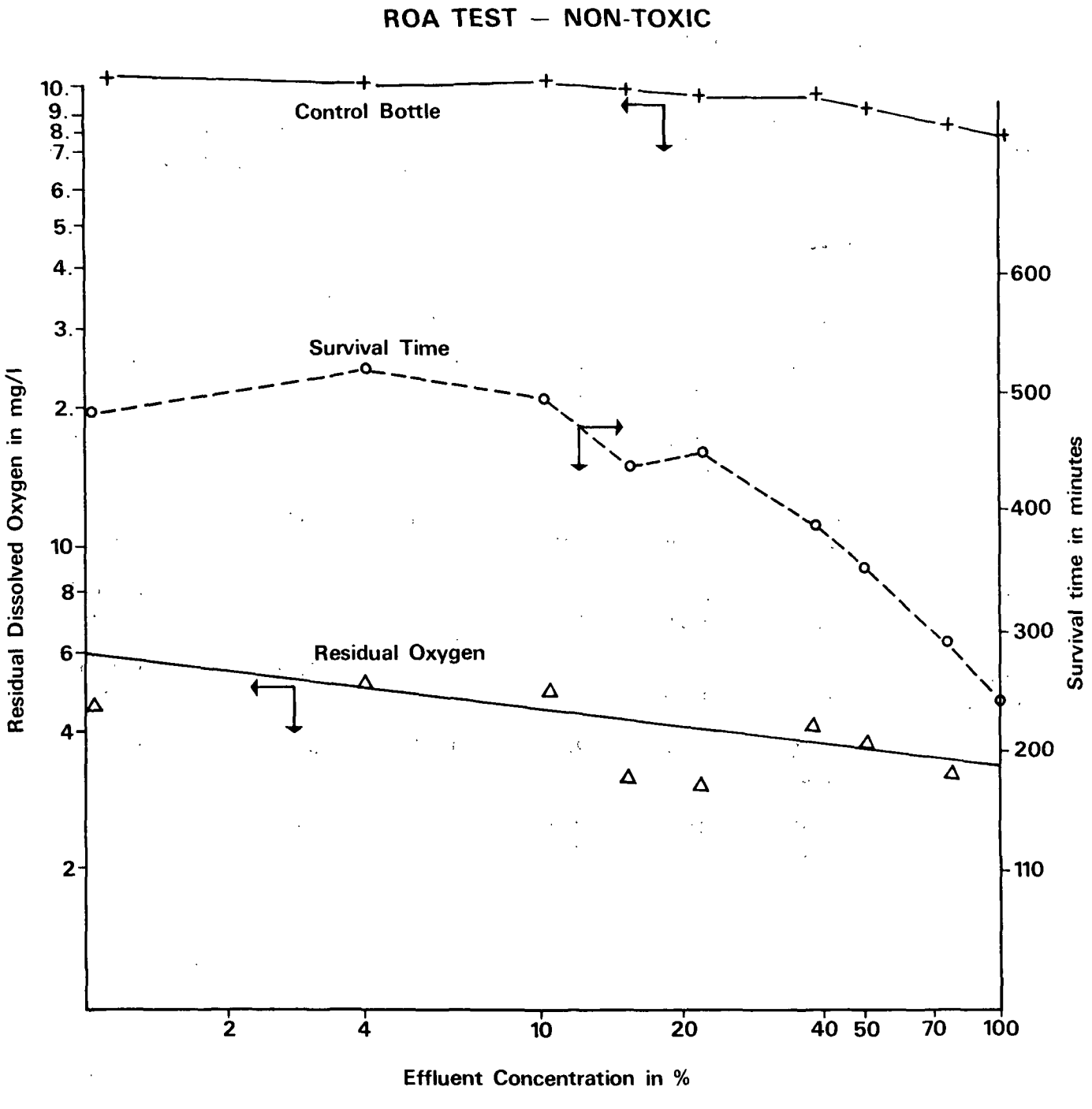

Figure 2. ROA curves for a representative nontoxic effluent.

test. Thus, no information on acute toxicity could be determined for this effluent. Figure 3 presents the plotted curves for this data set. One approach to eliminating BOD interference was tested, and the results are discussed later in this report.

TABLE IV

DATA GENERATED FOR A MILL EFFLUENT WITH EXCESSIVE BIOCHEMICAL OXYGEN DEMAND

Effluent Concentration	$\bar{x}$ Residual Oxygen, ppm	Start Temp., °C	$\bar{x}$ Elapsed Time, min	$\bar{x}$ Fish Wt./Bottle, g	$\bar{x}$ Fish Loading, g/L	$\bar{x}$ O_2 Uptake Rate,[a] mg O_2/g fish/min
0	0.95	23.5	397	1.09	3.54	--
Blank	7.8	23.5	525	--	--	--
4%	0.76	23.5	254	1.13	3.65	--
Blank	5.3	23.5	434	--	--	--
10%	0.55	23.5	240	1.02	3.31	--
Blank	4.6	23.7	331	--	--	--
15%	0.39	23.5	206	1.09	3.55	--
Blank	3.4	23.5	260	--	--	--
21%	0.31	23.5	131	1.16	3.78	--
Blank	3.0	23.6	166	--	--	--
37%	0.33	23.8	138	1.18	3.80	--
Blank	2.1	24.0	170	--	--	--
50%	0.27	23.8	98	1.36	4.41	--
Blank	2.7	23.8	107	--	--	--
75%	0.26	24.2	68.8	1.24	4.02	--
Blank	0.40	24.2	76			--
100%	0.37	24.8	48	1.26	4.10	--
Blank	0.3	24.8	54	--	--	--

[a]Oxygen uptake rates not discernible because of BOD interference.

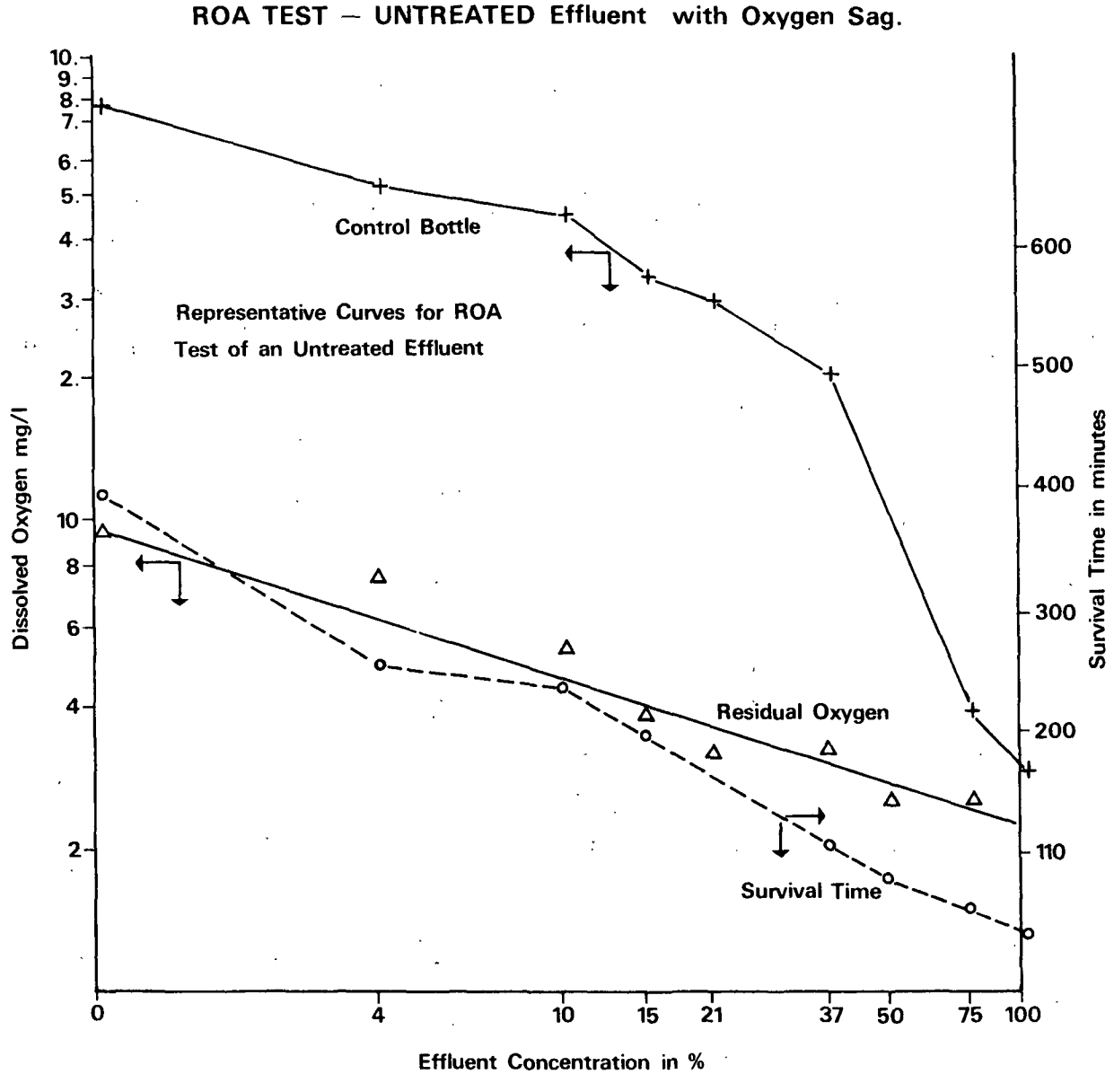

Figure 3. Plotted curves for an ROA with BOD interference.

Elapsed Time Values

The principal application of the ROA is to produce a threshold level value response for an acutely toxic effluent. However, it is quite likely that a biologically treated effluent may not be acutely toxic. In the 62 assays included in Appendix II, 27 did not produce a TLV, indicating that these were not acutely toxic. Five others did not produce a TLV because of excessive BOD which lowered residual oxygen levels, and two others had questionable results. Another response, evident in the fish during an ROA, was a fluctuating elapsed time to death value. This phenomenon was investigated further to determine whether elapsed time might indicate a sublethal response for some effluents.

For those assays which indicated no toxicity, data were evaluated for differences in elapsed time. Elapsed time is the time in minutes from the fish's introduction to the effluent solution to the time of its death. It is logical to expect that the elapsed time would be a function of temperature, fish size, and amount of oxygen available to be consumed (in a nontoxic effluent). If one assumes a relatively constant oxygen uptake rate or fish metabolic rate, the elapsed time to the death of the fish should be constant for any given set of temperature and initial oxygen levels. If the elapsed time changes in the presence of an effluent, it might reasonably be assumed that the change was due to some property of the effluent which could be described as a sublethal effect. Fish in toxic concentrations of effluents or other compounds characteristically have a shorter survival time than do those in clear water control bottles.

In Table V elapsed time differences for six of the assays included in Appendix II were tested using Student *t* test statistics. These six assays were of

toxic compounds (compounds which produced a TLV). Elapsed time to death for fish in the controls was tested against elapsed time to death for fish in the highest effluent concentration. Differences of as much as 55 fold were evident, and all six toxic compounds showed a significant difference between the elapsed times tested.

Difference in elapsed time occurred for some of the nontoxic effluents or effluents which did not produce a TLV. If this observation was valid, elapsed time may indicate a sublethal stressor which acts to speed up metabolic activity but does not influence residual oxygen levels. There is a need for a rapid dependable sublethal assay as well as for a rapid acute toxicity assay. Table V also includes comparative data summaries for 19 sets of data generated by ROA for 19 nonacutely toxic effluents. The elapsed times for control vs. 100% effluent concentrations were tested with the t test, which indicated that 10 of the effluents showed no difference in elapsed time. Of the remaining effluents, nine showed significantly different elapsed times, but seven of these nine also showed significantly different levels of dissolved oxygen in the bottles at the start of the test. This likely had an effect on elapsed time. One of the nine showed significantly different temperatures between the two concentrations, and it was disregarded. Only one of the 17 samples showed a significant difference in elapsed time with all other pertinent variables being equal.

For this large data base elapsed time did not indicate the presence of sublethal toxicity for any of the compounds or effluents tested. While it is possible that there were no sublethal effects this is not likely, especially in the case of the pure compounds pentachlorophenol and dehydroabietic acid which were

TABLE V

A SUMMARY OF ELAPSED TIME DATA FOR RESIDUAL OXYGEN ASSAYS

Effluent Number[a]	Effluent Type	Control: $\bar{x}$ Initial Oxygen, mg/L	Control: $\bar{x}$ Elapsed Time, min	Control: $\bar{x}$ Starting Temp., °C	Control: $\bar{x}$ Fish Weight, g	Highest Concentration Tested: $\bar{x}$ Initial Oxygen, mg/L	Highest Concentration Tested: $\bar{x}$ Elapsed Time, min	Highest Concentration Tested: $\bar{x}$ Starting Temp., °C	Highest Concentration Tested: $\bar{x}$ Fish Weight, g	DF	t[b]
				Nontoxic Effluents							
59	Unbleached kraft treated	7.4	225	24	1.51	7.5	175	25	1.7	20	2.40 n.s.
60	Bleached kraft treated	8.4	316	25	1.17	6.4	125	24	1.43	17	16.29**
61	Unbleached kraft treated	8.5	216	22	2.42	8.2	197	22	2.32	18	1.07 n.s.
62	Unbleached kraft treated	8.3	201	21	2.74	7.6	167	22	2.57	20	2.12 n.s.
46	Lab bleached kraft untreated	8.02	104	26	3.7	5.8	76	26	2.7	8	2.43 n.s.
47	Lab bleached kraft untreated	8.4	127	26.5	2.5	5.9	55	26.5	4.1	17	2.94 n.s.
10	Bleached kraft untreated	7.56	203	23	1.38	6.6	84	25	1.49	18	7.3**
11	Bleached kraft treated	7.7	175	23	1.5	4.8	194	23	1.64	18	3.49**
36	Lab bleached sulfite untreated	7.9	325	21	1.37	8.0	158	21	1.72	10	5.6**
2	Unbleached kraft treated	11.7	729	15	0.69	8.0	471	22	0.79	16	3.2**
3	Unbleached kraft treated	12.5	263	23	1.88	8.3	188	24	1.72	19	3.98**
12	Unbleached kraft treated	5.3	156	25	1.56	6.7	154	27	1.75	20	2.2 n.s.
17	Lab soda treated	6.5	228	24	1.19	7.7	275	23	1.23	20	1.54 n.s.
20	Bleached kraft treated	8.5	362	20	2.4	5.4	136	21.5	1.88	18	7.25**
23	Bleached kraft treated	9.6	383	21	1.96	8.0	198	20.0	1.24	18	7.38**
33	Bleached kraft treated	11.5	493	18	1.82	7.5	239	21	1.76	17	7.03**
18	Lab soda treated	5.0	248	22	1.07	7.7	322	25	1.29	20	2.73 n.s.
41	DHA	8.3	509	17.3	3.17	8.2	426	18.6	2.12	18	1.38 n.s.
42	DHA pH 6.5	9.1	621	17.0	1.38	9.2	470	15.8	1.1	13	2.3 n.s.
				Toxic Effluents							
43	DHA pH 7	8.0	372	20.0	1.69	8.2	124	19.5	1.5	17	12.5**
56	PCP	5.1	97	25.7	1.83	6.9	11	27.0	1.5	10	6.57**
44	DHA pH 8	8.0	352	22.0	2.1	7.7	101	21.0	1.94	18	6.12**
4	Lab bleached kraft untreated	8.5	192	19.5	1.78	10.2	105	22.0	1.94	19	5.75**
19	Bleached sulfate untreated	7.3	375	21.5	1.09	6.8	119	21.5	1.38	14	17.68**
21	Bleached sulfate untreated	6.2	304	19.8	1.29	6.7	81	20.0	1.5	10	33.9**

[a]Number as listed in Appendix.
[b]t** = significant at P = 0.01. n.s. = not significant.

tested at concentrations within 10% of their LC50 (determined by conventional assays). Thus, it appears that elapsed time in the ROA does not function as a sublethal stress indicator. This may be due to the relatively short exposure times involved or it may simply be evidence that the toxicants involved do not affect the fish's metabolic rate significantly more than do the test conditions themselves (i.e., handling, confinement, etc.).

Effects of Temperature on Elapsed Time

While elapsed time may not contribute to an evaluation of chronic stress, it is an important variable in this test. For the 62 assays conducted under this program the mean elapsed time ranged from 10 minutes to more than 850 minutes (14.1 hours). Occasionally survivors in controls or nontoxic effluents survived for longer than 24 hours. For practical application of this test a six-hour response time (or less) is most desirable to fit the conventional 8-hour work shift. This would allow adequate setup time and avoid a temptation to rush the results.

During this study, elapsed time could be manipulated by either increasing the temperature at which the tests were run or by increasing the loading rates of fish per bottle.

Loading rates were not extensively investigated as part of this project. However, other researchers have determined that loading rates do not affect the TLV (5). Data have not been presented in the literature for effects of loading rates on elapsed time except to indicate a relationship. This information is also not available from this study because of the narrow range in the size of the fish used. Some preliminary tests were done with increasing loading rates by using multiple fish in some bottles, and shorter elapsed times were observed.

A comparison of averaged fish weights and averaged elapsed times was inconclusive so some individual values were compared for assays which appeared to have the widest range of fish sizes. The results are included in Table VI. Fish weights and elapsed times for individual fish from the control bottles for four different assays were tested by a simple regression analysis. For data set A, a moderate correlation between fish size and elapsed time was indicated ($r^2 = 0.77$). For data set D a better correlation was found ($r^2 = 0.82$). But for data set C no relationship was apparent ($r^2 = 0.158$). In data set C the fish were of a uniform size and weight and the elapsed times were more variable.

For the normal range of sizes found for adult fatheads, size differences will not likely affect elapsed times significantly unless two or three fish are used per bottle.

The effect of temperature on elapsed time was much more apparent in the data available. Table VII includes data selected to represent ROA's conducted over a wide range of starting temperatures. To avoid interactions with toxicants only the elapsed times of the controls were considered (high concentration data are included for reference). As an example of the magnitude of the effect observed, assay No. 25 with an average fish weight of 1.5 grams and a starting temperature of 14.5°C had an average elapsed time of 514 minutes for the control. Meanwhile, assay No. 59, also with 1.5 grams fish but with a starting temperature of 24°C, had an average elapsed time of 225 minutes. When a linear regression was calculated for all the assays represented in Table VII ($n = 15$) an r^2 fit of 0.791 was obtained. This correlation was improved when elapsed time and temperature were compared for fish of similar weights (< 2 grams, $n = 9$), and an r^2 value of 0.93 was obtained.

TABLE VI

FISH WEIGHTS (GRAMS) AND ELAPSED TIME (MINUTES) COMPARED FOR INDIVIDUAL FISH IN CONTROL CONCENTRATIONS FOR FOUR REPRESENTATIVE ROA's

	Fish Weight, g	Elapsed Time, minutes	Starting Temp., °C
Data set A	2.03	245	22
	2.16	226	
	3.23	155	
	2.22	236	
	1.79	256	
	4.45	137	
	1.83	280	
	3.77	128	
	3.41	193	
	2.62	155	
$r^2 = 0.77$			
Data set B	2.95	156	22
	1.72	268	
	2.52	174	
	2.44	224	
	1.70	265	
	1.87	243	
	3.77	146	
	3.31	215	
	1.93	243	
	1.93	230	
$r^2 = 0.70$			
Data set C	1.38	212	24
	1.67	161	
	1.17	171	
	1.39	259	
	1.58	179	
	1.22	230	
	1.62	199	
	1.17	324	
	1.83	229	
	2.12	192	
$r^2 = 0.15$			
Data set D	2.49	112	22
	2.67	115	
	1.77	166	
	1.52	168	
	1.45	186	
	2.60	115	
	2.41	116	
	1.84	166	
	3.09	117	
	2.25	117	
$r^2 = 0.82$			

TABLE VII

TEMPERATURE AND ELAPSED TIME FOR RESIDUAL OXYGEN ASSAYS

		Control Concentration			Highest Concentration Tested		
No.	Effluent Description	$\bar{x}$ Fish Wt., g	$\bar{x}$ Temp., °C	$\bar{x}$ Elapsed Time, min	$\bar{x}$ Fish Wt., g	$\bar{x}$ Temp., °C	$\bar{x}$ Elapsed Time, min
34	PCP	1.7	10	> 854	1.9	21	68
7	Lab kraft treated	2.5	19	167	2.34	15	253
41	DHA	2.5	16	523	2.1	18.5	439
42	DHA	1.38	15.5	577	1.1	15	470
57	PCP	1.36	26	195	1.4	28	33
25	Lab borax untreated	1.5	14.5	514	1.35	23	37
31	Bleached kraft untreated	2.1	15	617	1.6	17	89.7
23	Bleached kraft treated	1.9	21	383	1.2	20	198
33	Bleached kraft treated	1.8	18	493	1.8	21	239
59	Unbleached kraft treated	1.5	24	225	1.7	25	175
61	Unbleached kraft treated	2.4	22	216	2.3	22	197
2	Unbleached kraft treated	0.7	15	729	0.8	22	471
6	Lab bleached kraft treated	2.1	19.5	199	2.5	20	223
8	Lab bleached kraft treated	2.2	22	139	1.9	25	163
12	Unbleached kraft treated	1.6	25	156	1.7	27	154

All data $r^2 = 0.79$, $n = 15$ $\quad$ $r^2 = 0.25$, $n = 15$

Data for fish < 2 grams $r^2 = 0.93$, $n = 9$

From the information available it is apparent that operating temperatures of 20-25°C are preferable when conducting an ROA. Temperatures in this range speed up the test but do not seem to affect the threshold limit. In Table VIII several sets of effluents are listed which were obtained from identical sources but tested at different times and under slightly differing temperatures. The difference in temperatures between the paired tests did not appreciably influence the results. For example, the temperatures of the tests for effluents 25 and 29 differed by as much as 5°C, but the TLV was 12% for both ROA's.

TABLE VIII

TEMPERATURES AND EFFECTS ON ROA THRESHOLD LIMITS

	Effluent Assayed	TLV, %	Start Temp.,[a] °C
4	Lab kraft untreated	23	24-20
5	Lab kraft untreated	27	19-22
21	Bleached sulfite untreated	3.5	20-21
22	Bleached sulfite untreated	4.2	18-19
25	Lab borax untreated	12	15-23
29	Lab borax untreated	12	19-20
34	PCP	1.2 ppm	9-21
35	PCP	1.1 ppm	19-21

[a]Temperature range for all concentrations.

Oxygen Uptake Rates

Another type of information which can be obtained by conducting a residual oxygen assay is the oxygen uptake rate. By measuring elapsed time, fish weight, and amount of oxygen consumed, an estimate of oxygen uptake can be calculated as milligrams O_2 per gram of fish per minute. In Table IX oxygen uptake rates for a selection of toxic and nontoxic effluents and compounds are presented. Oxygen uptake rates were corrected to account for oxygen consumed by spontaneous uptake by the effluent. The uptake rates for the control fish were compared with the uptake rates for the fish exposed to the highest concentration tested. These data were evaluated by the Student t test. Test temperatures and weight of the test fish are also included in Table IX.

The oxygen uptake rate is a function of the animal's metabolic activities and is affected by the size of the fish, the temperature of the surrounding water, and the amount of activity under way. It is also reasonable to assume that toxicants may either depress or enhance oxygen uptake rates. To investigate whether the ROA provides a convenient measurement of this response, toxic and nontoxic oxygen uptake rates were examined.

In Table IX seven toxic solutions with the greatest apparent differences in oxygen uptake rates were tested for similarity. Of the seven, only two were significantly different ($P = 0.01$).

Fifteen sets of data were compared for nonacutely toxic effluents. Of these, seven were significantly different and eight were not different. Of the seven which were different, two had differences in fish weights and two had differences in temperatures for the concentrations tested. The three remaining had no qualifications of the results obtained.

TABLE IX

A SUMMARY OF OXYGEN UPTAKE RATE DATA FOR RESIDUAL OXYGEN ASSAYS

Effluent Number[a]	Effluent Type	Control Concentration $\bar{x}$ Temp., °C	Control Concentration $\bar{x}$ Fish Wt., g	Control Concentration O_2 Uptake Rate	Highest Concentration $\bar{x}$ Temp., °C	Highest Concentration $\bar{x}$ Fish Wt., g	Highest Concentration O_2 Uptake Rate, mg/g/min	DF	t[b]
			Nontoxic Effluents						
59	Unbleached kraft treated	24	1.51	0.022	24.5	1.7	0.024	18	1.31 n.s.
61	Unbleached kraft treated	22	2.41	0.016	24.0	2.3	0.015	18	0.92 n.s.
62	Unbleached kraft treated	21	2.74	0.0153	22	2.57	0.0151	18	0.21 n.s.
60	Bleached kraft treated	25	1.17	0.021	24	1.43	0.0479	17	4.62**
24	Bleached kraft treated	20	2.4	0.0091	21.5	2.4	0.010	17	0.79 n.s.
23	Bleached kraft treated	21	1.96	0.012	20	1.24	0.0059	17	7.4**
10	Bleached kraft untreated	25	1.37	0.0252	25	1.49	0.0121	16	7.87**
11	Bleached kraft treated	24	1.5	0.0155	21.5	1.63	0.0114	18	2.6 n.s.
12	Unbleached kraft treated	27	1.75	0.0217	24.5	1.56	0.023	17	1.38 n.s.
9	Lab bleached kraft treated	24	2.21	0.0215	20	2.45	0.0174	16	4.27**
46	Lab bleached kraft untreated	26	3.7	0.0202	26	2.73	0.0312	6	3.37 n.s.
26	Bleached sulfite treated	19	1.66	0.0037	19	1.53	0.0210	14	8.79**
33	Bleached kraft treated	18	1.82	0.0124	21.5	1.76	0.0181	16	3.89**
42	DHA	15.5	1.38	0.0068	15	1.11	0.012	8	4.57**
45	DHA	23	2.37	0.0167	24	2.08	0.018	18	1.62 n.s.
			Toxic Effluents						
56	PCP	27	1.56	0.0269	27	1.47	0.041	8	2.86 n.s
43	DHA	20	1.69	0.0122	20	1.55	0.0215	17	4.37**
55	PCP	26	1.86	0.0266	27	1.54	0.0162	8	3.31 n.s.
21	Bleached kraft untreated	21	1.29	0.0147	20	1.50	0.0103	8	2.25 n.s.
22	Bleached kraft untreated	19	1.5	0.0143	20	1.95	0.0125	7	0.90 n.s.
5	Lab bleached kraft untreated	22.5	2.26	0.0215	22.5	1.92	0.0246	17	2.33 n.s.
14	Lab soda untreated	25	1.2	0.0232	25	1.56	0.0116	13	5.78**

[a]Number as listed in Appendix IV.
[b]t** = significant at P = 0.01.

Of the 22 sets of oxygen uptake rates compared there was a difference between the control and the highest exposed concentration for four. This is not a consistent enough response to establish a convincing argument for the universal use of this variable as a stress indicator. It may be true that these four materials were the only ones tested which had a sublethal effect on fish. Or, it may be that there are some groups of compounds in pulp mill effluents which do have an effect on fish metabolic rates and others which, while toxic, do not cause changes in oxygen uptake rates. Available literature on toxicity of pulp and paper mill effluents does not provide any useful information on this topic. Additional work with known sublethal toxicants is required to further clarify this issue.

II. APPLICATION OF THE ROA TO EFFLUENTS AND EFFLUENT COMPOUNDS

During this evaluation of the ROA a large variety of effluents and compounds was subjected to analysis. As a result it is possible to evaluate the application of the test to the different conditions encountered for a broad range of effluents. Table I lists the types of effluents surveyed, and Table X describes the results obtained for these categories of effluents.

Of the 62 effluents or compounds used, eight could not be tested or did not produce results because of the BOD problems previously discussed. These included both untreated and biologically treated effluents. However, the majority of problem effluents were those having no treatment or just enough pretreatment to establish a microbial community in the sample.

All of the nontoxic mill effluents and 10 of the 14 nontoxic lab effluents were biologically treated. Only one of the biologically treated effluents tested gave a toxic response, and this was at a very high concentration (82%).

TABLE X

RESPONSE TO ROA BY EFFLUENT TYPE

	Treated	Untreated	Total
Number of effluents from mill sources			
Kraft bleached	5	2	7
Kraft unbleached	6	3	9
Sulfite bleached	2	4	6
			22
Number of mill effluents not testable (due to high BOD or other complication)			
Kraft bleached		2	2
Kraft unbleached	1	4	5
Sulfite bleached	1		1
			8
Number of mill effluents producing a TLV (toxic response)			
Kraft bleached		0	
Kraft unbleached	1		1
Sulfite bleached		4	4
			5
Number of mill effluents not producing a TLV (nontoxic response)			
Kraft bleached	8		8
Kraft unbleached	5		5
Sulfite bleached	1		1
			14
Lab effluents with a toxic response			
Kraft bleached		2	2
Sulfite bleached			
Soda and soda borate bleached		5	5
			7
Lab effluents with nontoxic response			
Kraft bleached	4	2	6
Sulfite bleached		1	1
Soda and soda borate bleached	7		7
			14
Pure compounds with toxic response		14	14
Pure compounds with nontoxic response		5	5
			19

Untreated mill effluents were either toxic or not testable. For laboratory effluents three untreated samples were not toxic, but these were produced using experimental nonconventional bleaching methods.

Altogether, there were no unusual patterns of response to the ROA analysis by effluent type or source that would indicate limits to the application of this test. Pure compounds were the easiest to test because competition for oxygen was usually not a problem, and more information on type and concentration of toxicants present was available. In spite of this, the ROA probably has its greatest potential use as a quick, on-site monitoring tool for mill use.

III. THE ROA AS A TOXICITY TEST

Correlation with LC50

One of the most useful applications of the ROA test would be as a predictive tool for a conventional LC50 bioassay value. The LC50 is an estimate of the concentration of test material at which 50% of the exposed animals survive. This assay normally requires an exposure period of 96 hours or more, and a six-hour ROA would provide a much quicker response time if it reflected an LC50 response.

During this study numerous effluents assayed by the ROA were also assayed by conventional bioassay methods to produce an LC50. In Table X the available LC50 and TLV comparison data have been selected and summarized. A linear regression for these data is also summarized in Table XI.

For the 17 toxic wastes assayed, the ROA indicated higher toxicity than the LC50 for eight compounds and lower toxicity for nine compounds. One waste gave the same result with both assays. Linear regression analysis produced an r^2 of 0.848 for a correlation of all TLV's with all LC50's. A similar number ($r^2 = 0.83$) was

TABLE XI

THRESHOLD LIMIT VALUES AND CORRESPONDING LC50's

Effluent Number	Type	TLV	96-Hour LC50
3	Unbleached kraft treated	82%	> 100%
34	PCP	1.2 ppm	0.19 ppm Mean of 3
35	PCP	1.1 ppm	0.19 ppm Mean of 3
29	Lab soda untreated	11.5%	33%
13	Lab soda untreated	18%	6.1%
14	Lab soda untreated	14%	5.9%
22	Bleached sulfite untreated	4.2%	3.4% Mean of 4
20	Bleached sulfite untreated	2.2%	3.4% Mean of 4
21	Bleached sulfite untreated	3.5%	3.4% Mean of 4
25	Lab borax untreated	12%	33%
26	Bleached sulfite treated	None	36.0% Mean of 3
29	Lab soda untreated	12%	33%
33	Bleached kraft treated	None	89.7%
43	DHA pH 7	2.25 ppm	8.8 ppm Mean of 4
44	DHA pH 8	6-8.4 ppm	22.0 ppm Mean of 4
45	DHA pH 9	33 ppm	38.3% Mean of 4
57	PCP	0.85 ppm	0.19 ppm Mean of 3

Linear regression correlations:

all TLV with all LC50 $r^2 = 0.85$ $n = 15$

all effluent TLV with LC50 $r^2 = 0.83$ $n = 9$

mill effluent TLV with LC50 $r^2 = 0.99$ $n = 4$

lab effluent TLV with LC50 $r^2 = 0.71$ $n = 5$

pure compound TLV with LC50 $r^2 = 0.87$ $n = 6$

DHA TLV with LC50 $r^2 = 0.76$ $n = 3$

PCP TLV with LC50 $r^2 = 0.50$ $n = 3$

obtained when only effluents were evaluated and also for pure compounds when they were tested separately (r^2 = 0.875). When mill effluent data were listed, an r^2 of 0.999 was calculated; however, only four numbers were available.

Other researchers using the ROA obtained results comparable to LC50's obtained from 96-hour exposures. Vigers saw no significant difference between the TLV and the 96-hour LC50 for rainbow trout exposed to sodium pentachlorophenate (5). McLeay (4) found that "significant responses" by rainbow trout to DHA, zinc, phenol, and lindane occurred at concentrations near or lower than the 96-hour LC50. However McLeay did not use the TLV approach to analyze the ROA results.

Results from the present study for PCP (Table XII) show identical TLV and LC50 values as do the borax pulping effluents, untreated sulfite effluents, and DHA at pH 9. Most comparisons showed the same relative response range for both assays.

TABLE XII

SUMMARY OF RESULTS FROM PENTACHLOROPHENOL ASSAYS

	Effluent	TLV, ppm	LC50, ppm	$\bar{x}$ Start Temp., °C		Average Fish Wt./Container, g	Loading, g/L
				Control	100%		
34	PCP	1.2	0.19	10	21	1.90	6.1
35	PCP	1.1	0.19	20	21	0.90	2.8
57	PCP	0.85	0.19	26.4	28.1	1.41	4.5

In no case did the ROA indicate that an effluent was toxic in contrast to an LC50 which indicated that it was not. One effluent tested gave a negative test result for the ROA but was found to be quite toxic (36% mean of 3 assays) when tested by conventional assays. This test had marginally acceptable blank bottle response levels as well. The reason for this discrepancy is not known, and no other effluent or compound gave this type of result.

The reproducibility of this assay was quite good. The best reproducibility data are shown by the three pentachlorophenol assays which were conducted at three separate times but produced very similar threshold limit values of 1.1, 1.2, and 0.85 ppm. Thus, it would appear that by conducting an LC50/ROA comparison to calibrate the ROA a mill could thereafter collect frequent inexpensive toxicity data by using the ROA alone or by shipping a small volume of effluent to an analytical testing service.

Effect of Fish Loading Rates and Temperature on TLV

The effects of temperature on the ROA have been mentioned previously but bear some repetition. For fathead minnows, changing the temperature (with appropriate acclimation to avoid temperature shock mortalities) changes the elapsed time to death of the fish. This affects the time it takes to perform the assay but does not appear to affect the threshold level for a toxic effluent.

Other investigators (6) have seen changes in response levels associated with increased temperatures for rainbow trout and coho salmon. However, these species are cold-water fresh and normally become stressed with increasing temperatures.

The loading rate of fish weight per volume of effluent is a variable which can be controlled during an ROA. McLeay (4) found that while comparing loading rates of 1.1 g/L and 5.3 g/L some increased sensitivity to bleached kraft mill effluent by coho salmon was observed at the low loading rate. However, McLeay's experiment utilized 3.75-liter jars and very long exposure times, and the differences in sensitivity were still not large.

Loading rates in the present study varied according to the size of variations of adult fathead minnows. Typical loading rates of 3-7 g/L were preferred because of the shorter assay times involved.

The range of loadings in Table XII varies from 2.8 g/L to 6.1 g/L but no appreciable difference in the TLV resulted. It is most likely that the adult fathead minnows locally available will produce a range of loading rates that will not affect the application of this test to mill effluents using BOD bottles.

Previous Exposure Assays

During this experiment to evaluate the ROA procedure the question of exposure time was considered. This issue has two practical aspects. First, does the five- or six-hour exposure to toxicants in the ROA test contribute to a different response than the longer exposure for the 96-hour test? According to the previous comparisons with LC50's, this would not appear to be the case. Second, would prior exposure to a toxicant affect the threshold level measured by the ROA?

To address this issue a population of fathead minnows was exposed to 0.1 mg/L of pentachlorophenol for periods varying from 0 to 72 hours. This concentration is about half of the LC50 concentration as determined by static acute bioassays for fathead minnows. A control population was handled in a way similar to the exposed fish but were exposed only to water. The unexposed fish were assayed at 72 hours.

The results of this experiment are summarized in Table XIII. Fish exposed to PCP for 24 hours were more sensitive than those not previously exposed. The TLV was further reduced by half following 48-hour exposure. A pattern of increasing susceptibility for fish under stress had become apparent. However, the fish exposed for 72 hours were less sensitive, and the range of concentrations used did not include the threshold level, which was greater than 0.2 mg/L. No verification of toxicant concentrations by gas chromatography was done for the experiment, and the possibility of error in solution mixing could account for the unexpected response by the 72-hour fish.

TABLE XIII

SUMMARY OF RESULTS FOR PREEXPOSURE AND STORAGE EXPERIMENTS

Test	TLV
Exposure Results	
Exposed to PCP for 0 hours	0.85 mg/L
Exposed to PCP for 24 hours	0.32 mg/L
Exposed to PCP for 48 hours	0.15 mg/L
Exposed to PCP for 72 hours	> 0.2 mg/L
Storage Results	
Sulfite effluent fresh	2.2%
Sulfite effluent stored 48 hours	3.5%
Sulfite effluent stored 96 hours	4.2%

Apparently, stressed fish do become more sensitive to toxicity. Because of the short response time, the ROA could be used in conjunction with other exposure methods to conduct stress limit tests and long-term exposure tests. Little information was produced, however, that was related to the effect of exposure time on the sensitivity of the ROA. This question may merit further consideration.

Effluent Storage Assay

Another side benefit of the ROA to toxicological studies acrues from its short response time. The effect of handling, sample collection procedures, short-term, long-term, or temporary changes in effluent composition can all be measured more accurately than by the 96-hour conventional exposure assay.

An example of this application is an effluent storage study conducted for a mill effluent. A sample of untreated sulfite mill effluent was collected and a portion assayed. The remaining portions of the sample were stored, and over a four-day period an assay was periodically conducted to measure changes due to storage.

This type of information cannot be reliably obtained using a 96-hour exposure test, because variables affecting stored effluents will also affect the effluent in the bioassay over the long exposure period.

The results from this application of the ROA are also included in Table XII under "storage results." The fresh sample had a TLV of 2.2%. After storage for 96 hours the toxicity of the sample had decreased somewhat to a TLV level of 4.2%, a change probably undetectable using a conventional bioassay.

IV. ULTRAVIOLET TREATMENT TO CONTROL BOD INTERFERENCE

One serious limit to the use of the ROA test for some effluents such as leachates and partly treated mill effluents may be the oxygen demand interference from BOD which has been described earlier in the report. If a method could be found to eliminate this problem, this assay would have wider use and greater precision.

Since it is the activity of microbial organisms which oxidizes organic compounds and depletes oxygen levels, a method to inactivate or kill bacteria was considered. Autoclaving and chlorination or other chemical treatments would have a severe impact on the quality and composition of the effluents. While some chemical compounds may also be affected by the technique chosen, it was decided that ultraviolet (UV) light may be the least damaging, effective way to kill microbes in effluents in spite of color and turbidity problems.

Two samples of untreated kraft mill effluent were treated with UV light using a 15-inch commercial aquarium disinfection unit. After frequent intervals, samples were collected, sealed in BOD bottles (without fish), and monitored for oxygen consumption. The results of this exercise are included in Table XIV.

Effluent 1 was a bleach plant effluent from a bleached kraft mill, and effluent 2 was whole mill effluent from an unbleached kraft mill. Both effluents were dark and highly turbid. Effluent 2 had a light penetration of only 6% (at 460 millimicrons) after being diluted to 25% by volume.

Effluent 1 was not diluted but was UV tested for up to five hours. The UV-treated effluent consumed slightly more oxygen than the untreated samples. The UV treatment did not prove effective for undiluted samples of this effluent.

For highly colored or turbid samples, UV treatment apparently can be employed on diluted samples only. It may be possible to assay the diluted samples and back calculate to obtain an estimate for undiluted samples. This step was not done during the present study and remains to be verified by the work of others.

TABLE XIV

EFFECT OF UV TREATMENT ON O_2 CONSUMPTION OF TWO UNTREATED KRAFT MILL EFFLUENTS

	Effluent 1					Effluent 2						
Elapsed Time in Sealed Jar	100% Untreated	100% 20 min UV	100% 1 hr UV	100% 2 hr UV	100% 5 hr UV	100% Untreated	10% 1 hr UV	10% 2 hr UV	25% Untreated	25% 1 1/2 hr UV	25% 3 hr Uv	25% 4 hr UV
1/2 hour	8	19	--	--	--	--	--	--	9.2	10.9	4.5	--
1 hour	--	--	--	--	12	17.7	9.3					
2 hour	--	--	--	--	--	31.6	12.0	2.7				
3 hour	--	--	--	19	--	40.6	18.6	10.9	15.7	16.0	21.2	17.1
4 hour	20	32	33	32	--	42.2	23.0	12.3				
5 hour	28	39	46	--	--	--	--	--	30.5	23.6	35.0	34.2
6 hour	--	--	--	--	--	--	--	--	46.0	34.5		
8 hour	--	--	--	--	--	--						
18 hour	--	--	--	--	91	--						
21 hour	90	89	89	94	--	--						
22 hour	--	--	--	--	--	--	--	--	100	100	100	100
% Transparency at 460 milli-microns	--	--	--	--	--	45	--	--	6	9	10	11
Flow rate	2 liters/min ——————→1					2.8 liters/min ——————→1						
Volume treated	4 liters					4 liters						

CONCLUSIONS

The residual oxygen assay has great merit and application as a quick-response, simple, and effective test for acute toxicity of effluents and compounds to fathead minnows. The ROA provides sensitive, reproducible results which can be obtained on site by mill personnel at a very low cost and with a minimum of equipment. This assay would allow for routine monitoring of effluents or wastewater acute toxicity as a wastewater treatment plant operating parameter. It could also produce a large data base with which to evaluate effluent impacts on receiving streams.

While a fair to good correlation exists for the threshold limit value with LC50's obtained by conventional fish assay methods, no clear sublethal response could be identified. Elapsed time to death appears to be more a function of temperature and fish loading than sublethal toxicity. Oxygen uptake rates were for the most part not significantly different except where differences were due to identifiable phenomena such as differences in temperature.

Competition for oxygen from BOD compounds remains a problem for some types of effluents and leachates. However, pretreatment with UV light may provide a solution.

Where applicable, the ROA should prove to be a versatile and inexpensive means of generating a large and useful data base relative to the acute toxicity of a number of mill effluents or effluent compounds.

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THE INSTITUTE OF PAPER CHEMISTRY

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APPENDIX I

LABORATORY DECHLORINATED WATER CHARACTERISTICS

Parameter	Mean	Range
Chlorides, mg/L	13.9	10.9-15.6
Calcium as $CaCO_3$, mg/L	18.1	14.6-22.9
Magnesium, mg/L	32.9	29.9-38.9
Alkalinity as $CaCO_3$, mg/L	22.8	13.0-38.5
Hardness, mg/L	78.6	65.8-104.8
Conductivity, micromho/cm	173	150-235
pH	7.9	6.6-9.9
Iron, mg/L	0.16	--
Total residual Cl	0.01	0.01-0.02
Ammonia	0.09	0.16-0.04

APPENDIX II

RESIDUAL OXYGEN ASSAY (ROA)

			Initial		Final	
Effluent			pH	Cond.	pH	Cond.
Date		Control				
Conc. %		1 Test Bottle				

	Initial		Final						
No.	DO, mg/L	Temp., °C	DO, mg/L	Temp., °C	Time Start	Time End	Time Elapsed	Fish Wt., g	Load, g/L

		Initial		Final	
		pH	Cond.	pH	Cond.
Conc. %	Control				
	1 Test Bottle				

	Initial		Final						
No.	DO, mg/L	Temp., °C	DO, mg/L	Temp., °C	Time Start	Time End	Time Elapsed	Fish Wt., g	Load, g/L

APPENDIX III

STEPWISE PROCEDURES AND MATERIALS NECESSARY TO CONDUCT AN ROA ON A MILL SITE

Apparatus

Instruments

Dissolved oxygen meter with BOD bottle probe and built-in stirrer

pH meter

Conductivity meter

Balance (for weighing fish)

Glassware/Plastic

Graduated cylinders - 250, 1000, 2000 mL

Beakers - several 1000-mL beakers

BOD bottles and glass stoppers (11 bottles/concentration tested)

4-Liter bottles, one per concentration

Large container to store dilution water at room temperature

Mixing rod for aeration

Fish

Stock of healthy acclimated fish

Net

Washing Procedure for Glassware

BOD bottles

1. Rinse with water
2. Rinse with chromic acid
3. Rinse with water
4. Rinse with 10% HCl

APPENDIX III (Continued)

Washing Procedure for Glassware (Contd.)

BOD bottles (Contd.)

5. Rinse with water
6. Rinse with dechlorinated or distilled water

Other glassware

1. Rinse with water
2. Wash with Alconox
3. Rinse with water
4. Rinse with 10% HCl
5. Rinse with water
6. Rinse with dechlorinated or distilled water

Sample Pretreatment

1. Adjust pH of samples to approximately 7 with HCl or NaOH
2. Aerate effluent and dilution water - saturate with compressed air or mechanically saturate. A useful mixer for mechanical saturation consists of a perforated stainless steel plate attached to a rod.

Analysis Procedures

1. Determine concentration range to be tested.

   Treated effluent concentration 0% effluent, 4% effluent, 10, 15, 21, 37, 50, 75, and 100%. (See Table attached to this Appendix for sample dilutions).

2. Set up BOD bottles in numerical order in groups of 11 bottles/concentration.
3. Record bottle numbers and concentrations on data sheets (See Appendix I for sample data sheet).
4. Prepare sample solutions, 4 liters for each test concentration. Mix solutions vigorously to aerate them.
5. Fill appropriate BOD bottles.

   Note. Save excess sample solutions for initial pH and conductivity readings. Record on data sheets.

APPENDIX III (Continued)

Analysis Procedures (Contd.)

6. Record DO and temperature readings for each bottle.

7. Add fish to 10 bottles in each concentration and cap immediately. The eleventh bottle capped is also a control bottle for biological and chemical oxygen consumption. Record start time when fish are added for each concentration.

8. Record time of death, final DO, and temperature readings when each fish dies. Death is determined by cessation of all movement, including that of the gill operculum.

9. When all the fish in a concentration are dead, record the final DO, temperature, pH, and conductivity for the control bottle.

10. Record final pH and conductivity in the last fish bottle to be measured for each concentration.

11. Weigh each fish and record on data sheet.

    (Pour each BOD bottle through a net to remove fish. Blot each fish to remove excess water.)

Calculations

12. Calculation of threshold limit value (TLV).

    a. Find mean final (residual) oxygen for each concentration. (Average values in "final DO" column on data sheet.)

    b. Plot mean residual oxygen on log-log paper against effluent concentration.

    c. Determine concentration at which line slope changes = TLV.

    d. Line fitting formulae can be used as appropriate in c.

Optional Calculations for Supplemental Information

13. Calculate elapsed time in minutes.

14. Calculate loading: mg of fish/liter (find average volume of BOD bottles).

15. Calculate corrected dissolved oxygen (CDO) consumed for each bottle.

    a. Initial DO of control - final DO of control = K factor.

    b. For each fish the CDO = initial DO - final DO - K factor.

APPENDIX III (Continued)

Optional Calculations for Supplemental Information (Contd.)

16. Calculate corrected oxygen uptake rate mg/L O_2/min/mg of fish CDO ÷ elapsed time ÷ weight of fish = corrected oxygen uptake rate.

APPENDIX III (Continued)

ROA DILUTIONS

(11 Bottles)

Concentration, %	Toxicant Volume, mL	Water Volume, mL
0	0	4000
4	160	3840
10	400	3600
15	600	3400
21	840	3160
37	1480	2520
50	2000	2000
75	3000	1000
100	4000	0

APPENDIX IV

LIST AND SUMMARY OF DATA FOR ALL ROA's

					Control Concentration					Highest Concentration				
	Effluent or Code	Date	TLV	LC50	$\bar{x}$ Temp., °C	$\bar{x}$ Fish Weight, g	$\bar{x}$ Elapsed Time, min	$\bar{x}$ O_2 Uptake, mg/g/min	Final O_2 in Blank, mg/L	$\bar{x}$ Temp., °C	$\bar{x}$ Fish Weight, g	$\bar{x}$ Elapsed Time, min	$\bar{x}$ O_2 Uptake, mg/g/min	Final O_2 in Blank, mg/L
1	Unbleached kraft untreated	3/29/78	?	N.T.	20.5	1.51	333	N.C.	No blank	21	1.87	136	N.C.	No blank
2	Unbleached kraft treated	4/18/78	None	> 100%	15	0.69	729	0.0107	10.8	22	0.79	471	0.072	7.1
3	Unbleached kraft treated	5/9/78	82%	> 100%	23	1.88	263	0.023	10.6	24	1.72	188	0.022	7.5
4	Lab bleached kraft untreated	5/24/78	23%	N.T.	19.5	1.78	192	0.021	8.3	22	1.94	105	0.030	8.9
5	Lab bleached kraft untreated	5/25/78	27%	N.T.	22.5	2.26	274	0.0215	8.5	22.5	1.92	107	0.0246	9.5
6	Lab bleached kraft treated	6/14/78	None	> 100%	19.5	2.1	199	0.017	7.8	20	2.5	223	0.017	8.0
7	Lab bleached kraft treated	6/15/78	None	> 100%	19	2.5	167	0.018	7.9	15	2.34	253	0.018	9.7
8	Lab bleached kraft treated	6/27/78	None	> 100%	22	2.2	139	0.021	7.9	25	1.9	163	0.030	9.3
9	Lab bleached kraft treated	6/29/78	None	> 100%	22	2.2	137	0.021	6.6	20	2.5	140	0.023	6.05
10	Bleached kraft untreated	7/7/78	None	N.T.	23	1.38	203	0.0252	7.3	25	1.49	84	0.0121	2.5
11	Bleached kraft treated	7/11/78	None	N.T.	23	1.50	175	0.0155	7.1	23	1.64	194	0.0144	3.7
12	Unbleached kraft treated	8/16/78	None	> 100%	25	1.56	156	0.0217	4.7	27	1.75	154	0.023	6.7
13	Lab soda untreated	8/22/78	18%	6.1	23.5	1.21	254	0.0214	6.1	22.5	1.14	78	0.0157	8.1
14	Lab soda untreated	8/24/78	14%	5.9	25	1.2	239	0.0232	6.0	25	1.56	61	0.0116	7.4
15	Lab soda treated	9/16/78	None	> 100%	23	1.2	280	0.025	8.6	21	1.5	224	0.0279	8.2
16	Lab soda treated	9/7/78	None	> 100%	24	1.1	227	0.0212	5.8	25	1.04	272	0.0265	7.1
17	Lab soda treated	9/12/78	None	> 100%	24	1.19	228	0.0220	5.4	23	1.23	275	0.0218	6.7
18	Lab soda treated	9/14/78	None	> 100%	22	1.07	248	0.017	4.8	25	1.29	322	0.0177	6.9
19	Bleached sulfite untreated	9/21/78	23%?	3.9%	21.5	1.09	375	0.016	3.7	21.5	1.38	119	0.035	7.8
20	Bleached sulfite untreated	9/25/78	2.2%	3.4%	20	1.28	445	0.012	7.1	23	1.03	66	0.050	4.4
21	Bleached sulfite untreated	9/27/78	3.5%	3.4%	19.8	1.29	304	0.0147	6.0	20	1.5	80.5	0.0103	5.4
22	Bleached sulfite untreated	9/29/78	4.2%	3.4%	24	2.21	282	0.0143	7.9	20	2.45	87	0.0125	6.7

APPENDIX IV (Continued)

LIST AND SUMMARY OF DATA FOR ALL ROA's

					Control Concentration					Highest Concentration				
	Effluent or Code	Date	TLV	LC50	$\bar{x}$ Temp., °C	$\bar{x}$ Fish Weight, g	$\bar{x}$ Elapsed Time, min	$\bar{x}$ O_2 Uptake, mg/g/min	Final O_2 in Blank, mg/L	$\bar{x}$ Temp., °C	$\bar{x}$ Fish Weight, g	$\bar{x}$ Elapsed Time, min	$\bar{x}$ O_2 Uptake, mg/g/min	Final O_2 in Blank, mg/L
23	Bleached kraft treated	10/23/78	None	> 100%	21	1.96	383	0.012	9.4	20	1.24	198	0.006	2.2
24	Bleached kraft treated	10/25/78	None	> 100%	20	2.4	362	0.009	9.1	21.5	1.88	136	0.010	3.0
25	Lab borax untreated	11/8/78	12%	33%	14.5	1.5	514	0.0127	10.2	23	1.35	37	0.0214	7.9
26	Bleached sulfite treated	11/14/78	None	36.0% Mean of 3	19	1.66	633	0.0037	4.6	19	1.53	195	0.0210	2.7
27	Lab soda/bleached treated	11/16/78	None	> 100%	20	1.4	493	0.0129	8.9	18	1.8	253	0.0125	4.0
28	Lab soda/bleached treated	11/28/78	None	> 100%	20	1.4	> 741	0.0054	9.5	22	1.8	96.5	0.031	2.7
29	Lab soda/bleached untreated	11/30/78	11.5%	33	23	1.47	443	0.015	10.1	20.5	1.42	101	0.022	10.4
30	Unbleached kraft untreated	12/18/78	None	26.5	19.2	1.74	1492	0.0047	11.1	20.5	1.92	64	0.036	0.05
31	Bleached kraft untreated	1/18/79	None	28.25	15	2.1	617	0.0094	8.7	17	1.6	89.7	0.0386	0.3
32	Bleached sulfite treated	1/24/79	None	34%	20	1.63	444	0.0111	8.15	20	1.82	31	0.125	0.3
33	Bleached kraft treated	2/15/79	None	89.7% Mean of 4	18	1.82	493	0.0124	11.2	21	1.76	239	0.0181	7.4
34	PCP	3/1/79	1.2 ppm	0.19 ppm (3 reps.)	10	1.7	> 854	0.007	10.2	21	1.9	68	0.019	8.4
35	PCP	3/8/79	1.1 ppm	0.19 ppm (3 reps.)	20	1.82	507	0.010	8.7	21	1.62	36	0.002	5.5
36	Lab bleached sulfite untreated	5/10/79	None	N.T.	21	1.37	325	N.C.	7.4	21	1.37	158	N.C.	4.0
37	DHA soft water	2/28/80	2.5 ppm	?	21	1.35	388	0.0197	9.1	22	1.51	312	0.0158	8.9
38	DHA commercial grade	3/6/80	None	?			118					214		
39	DHA soft water	3/6/80	Atypical	N.T.	21	2.0	255	0.0164	8.7	21	1.92	226	0.0199	8.9
40	DHA commercial grade	4/3/80	3.9	?	21	1.68	311	0.017	7.7	21	1.54	225	0.0161	8.2
41	DHA	11/4/80	> 5 ppm		17.3	3.17	509	0.0057	8.2	18.6	2.1	426	0.0076	8.4
42	DHA pH 6.5	11/13/80	> 5 ppm		17	1.38	621	0.0068	3.4	15.8	1.1	470	0.012	9.5

APPENDIX IV (Continued)

LIST AND SUMMARY OF DATA FOR ALL ROA's

	Effluent or Code	Date	TLV	LC50	Control Concentration					Highest Concentration				
					$\bar{x}$ Temp., °C	$\bar{x}$ Fish Weight, g	$\bar{x}$ Elapsed Time, min	$\bar{x}$ O_2 Uptake, mg/g/min	Final O_2 in Blank, mg/L	$\bar{x}$ Temp., °C	$\bar{x}$ Fish Weight, g	$\bar{x}$ Elapsed Time, min	$\bar{x}$ O_2 Uptake, mg/g/min	Final O_2 in Blank, mg/L
43	DHA pH 7	12/11/80	2.25 ppm	8.8 ppm Mean of 4	20	1.69	372	0.0122	7.9	19.5	1.5	124	0.0215	8.02
44	DHA pH 8	12/18/80	6-8.4 ppm	22.0 ppm Mean of 4	22	2.1	352	0.0115	7.9	21	1.94	101	0.0134	7.3
45	DHA pH 9	1/15/81	33 ppm	38.3 Mean of 5	23	2.37	271	0.0167		24	2.08	234	0.018	
46	Lab bleached kraft untreated	2/20/81	None	N.T.	26	3.7	104	0.0202	8.4	26	2.7	76	0.0312	2.8
47	Lab bleached kraft untreated	2/20/81	None	N.T.	26.5	2.5	127	0.026	8.6	26.5	4.1	55	0.023	4.1
48	Unbleached kraft untreated	3/5/81	None	N.T.	22	1.91	217	0.021	8.5	21.2	2.7	78	0.033	7.1
49	Alum and unbleached kraft untreated	3/5/81	50%	N.T.	21.8	3.6	145	0.0157	8.6	21.8	3.3	142	0.014	8.2
50	Alum and water	3/5/81	21%	N.T.	21.8	1.54	202	0.025	8.8	21.5	2.8	55	0.0115	10.2
51	Polymer and water	3/10/81	< 10%	N.T.	22	2.71	158	0.016	7.15	22	3.39	108	0.0097	8.5
52	Polymer and unbleached kraft untreated	3/10/81	None	N.T.	22	4.06	114	0.015	7.0	21	2.1	112	0.030	5.2
53	Polymer and water	4/2/81	< 4%	N.T.	21	1.2	296	0.018	8.5	21	1.24	124	0.016	8.3
54	Alum and water	4/2/81	None	N.T.	21	1.24	256	0.0228	8.0	21	1.75	203	0.0215	7.7
55	PCP-Exposure-48	7/8/81	0.15 ppm	N.T.	26	1.86	154	0.0266	5.0	27	1.54	18.4	0.0162	6.9
56	PCP-Exposure-24	7/7/81	0.32 ppm	N.T.	25.7	1.83	97	0.0269	6.5	27	1.5	10.8	0.041	6.3
57	PCP-Exposure-72	7/9/81	> 0.2 ppm	N.T.	26	1.36	91	0.0294	4.9	28	1.37	33	0.135	None
58	PCP-Exposure-0	7/9/81	?0.85 ppm	0.19 Mean of 3	26	1.36	195	0.0218	4.9	28	1.4	33	0.0938	5.3
59	Unbleached kraft treated	8/6/81	None	N.T.	24	1.51	225	0.022	7.5	25	1.7	175	0.024	6.7
60	Bleached kraft treated	9/16/81	None	> 100%	25	1.17	316	0.021	8.0	24	1.43	125	0.047	4.3
61	Unbleached kraft treated	11/25/81	None	N.T.	22	2.42	216	0.016	8.39	22	2.32	197	0.015	6.7
62	Unbleached kraft treated	2/16/82	None	N.T.	21	2.74	201	0.0153	8.5	22	2.57	167	0.0151	6.8

N.T. = Not tested.
N.C. = Not calculated.